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Phosphorylation of tobacco eukaryotic translation initiation factor 4A upon pollen tube germination

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ABSTRACT

Eukaryotic translation initiation factor eIF-4A is a member of the DEAD box family of RNA helicases and RNA-dependent ATPases. In tobacco, eIF-4A is encoded by a gene family with one isoform, eIF-4A8, being exclusively expressed in pollen. This pollen-specific isoform is a candidate for mediating translational control in the developing gametophyte. Here we show that eIF-4A is barely phosphorylated in mature pollen, but during pollen tube germination, two isoforms of eIF-4A become phosphorylated. Phosphoamino acid analysis indicated phosphorylation of threonine. In order to determine whether pollen-specific eIF-4A8 is among the phosphorylated isoforms, we raised transgenic tobacco plants overexpressing eIF-4A8 containing a histidine tag. Hereby, we could show that indeed eIF-4A8 is modified through phosphorylation. The biological relevance of the phosphorylation of eIF-4A is discussed.

INTRODUCTION

Eukaryotic translation initiation factor 4A (eIF-4A) is an RNA helicase with RNA-dependent ATPase activity, which, in conjunction with eIF-4B, is thought to remove secondary structures in the 5′-untranslated region (UTR) of the mRNAs. This facilitates the binding of the 40S ribosomal subunit to the mRNA and the subsequent migration of the preinitiation complex to the AUG start codon (1,2).

Translation is an important site of regulation of gene expression, and the initiation stage is the most commonly observed target for physiological control (2). In particular, the phosphorylation of initiation factors is thought to regulate translation rates (3). The regulation of translation by the phosphorylation–dephosphorylation of eIF-4E (4,5), of the eIF-4E-binding protein (6) and of eIF-2α (7–9) has been well documented. Concerning eIF-4A, however, there is no evidence for phosphorylation in yeast or in mammalian systems (3). In contrast, it was shown that eIF-4A becomes phosphorylated upon oxygen deprivation in maize root tips (10), and upon heat shock in wheat (11). In both cases, the phosphorylation of eIF-4A correlates with a reduction in translation rates, and thus, eIF-4A is a potential candidate for regulating translation initiation in plants.

The eIF-4A gene family has been studied extensively in tobacco (12–16). In tobacco leaves, there is a minimum of 10 expressed genes, which fall into two highly divergent gene families (NeIF-4A2 and 3; 13). This is different from the situation in yeast (17) and mouse (18), where duplicate genes code for identical or highly similar proteins. The large number and the divergence of the plant eIF-4A genes suggests that they may have plant specific functions or that they might translate various mRNAs with different efficiencies. One isoform, *NeIF-4A8*, was found to be specifically expressed in tobacco pollen, and it was proposed to be a possible candidate for mediating translational control in the developing male gametophyte (14).

During male germ line development in tobacco, the products of meiosis, the microspores, undergo a highly asymmetric mitotic division. The resulting bicellular microspores dehydrate and mature into metabolically dormant pollen grains (19). When the pollen grain lands on a compatible pistil, water is rapidly taken up, metabolism resumes at a high rate, and a pollen tube is formed. During the pollen tube growth a further mitotic division occurs. The now tricellular pollen forms an ~4 cm long pollen tube and the double fertilization typical of flowering plants takes place in the ovule (20). Gene expression during plant male germ line development has several unique aspects. First, many novel proteins must be synthesized at different stages of development (21,22). Second, metabolic rates vary strongly and rapidly over time (21,23,24). Third, the ionic environment is highly variable and, in particular during dehydration, specific proteins must be synthesized in a cytoplasm estimated to contain ~300 mM KCl (25). Thus, the highly specialized haploid phase of the male life cycle puts very specific and unusual requirements on the gene expression machinery. In contrast to well-studied animal systems, pollen carry out transcription in the haploid phase of the life cycle. Some of the mRNAs are translated directly, while others are thought to be stored and translated at various times after pollen tube germination (21,26,27). In fact, the first stage of pollen germination up to the second mitotic division can take place in the presence of RNA polymerase inhibitors, indicating that this initial phase can proceed through translation of RNAs synthesized before dehydration (23,28,29). An interesting example of translational regulation is provided by the pollen-specific *lat52* mRNA. The 5′-UTR of this mRNA enhances translation relative to control 5′-UTRs during the final stages of pollen maturation in a pollen-specific and strictly developmentally regulated manner (30). The mechanism underlying this pollen-specific translational enhancement is unknown, but it seems reasonable to assume that it is mediated through specific interactions between 5′-UTRs and

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regulatory proteins. The pollen-specific eIF-4A8 is an attractive candidate for mediating this selective translation.

In this paper, we characterize the isoforms of eIF-4A occurring in pollen. We demonstrate that eIF-4A is phosphorylated in tobacco and that pollen tube germination is accompanied by a rapid increase in the phosphorylation of eIF-4A.

MATERIALS AND METHODS

Plant growth conditions

Plants of *Nicotiana tabacum* cv. Samsun were grown in the greenhouse, under a 16:8 h light:dark cycle and a temperature of at least 18°C. Mature pollen was harvested using a 35 µm mesh filter connected to a vacuum cleaner. Pollen was germinated in 25 mM MES–KOH (pH 5.9), 0.3 M sucrose, 3.0 mM $Ca(NO_3)_2$, 1.6 mM H_3BO_3, 1.0 mM KNO_3, 0.8 mM $MgSO_4$, 30 µM $CuSO_4$, 0.1% (w/v) caseine hydrolysate and 1.5 µM quercetin (31). For *in vivo* labelling of phosphoproteins, 500 µCi $H_3{}^{32}PO_4$ (8500 Ci/mmol, Amersham, further referred to as $^{32}P_i$) was added per ml medium.

Two-dimensional electrophoresis/western analysis

Total soluble proteins were isolated by grinding and extracting in 50 mM Tris–HCl (pH 8.0), 1% Triton X-100, 1% β-mercapto-ethanol and 1% polyvinyl-polypyrrolidon (PVPP). The samples were desalted using Centricon 10 concentrators (Amicon). Samples (10 µg) of total protein were loaded on the acidic side of IEF tube gels [4.5% acrylamide/bisacrylamide (30:1.8), 8.3 M urea, 4.8% ampholytes (Bio-Rad, 5/7:3/10 = 2:1), 2% CHAPS] and run at 400 V for 4.5 h, followed by 0.5 h at 500 V. The protein was run in the second dimension using standard 10% SDS–PAGE after equilibration of the tube gel for 15 min in 62.5 mM Tris–HCl (pH 6.8), 1% DTE, 3% SDS and 10% glycerol. The proteins were transferred to nitro-cellulose (Schleicher and Schuell) and a 1:1000 dilution of rabbit anti-eIF-4A antibody (13) and horseradish peroxidase conjugates were used to detect eIF-4A proteins. To estimate the pI and MW, the two-dimensional SDS–PAGE standard of Bio-Rad was used in parallel gels.

Immunoprecipitation of eIF-4A

For immunoprecipitation of eIf-4A, pollen was extracted in a buffer containing 50 mM Tris–HCl (pH 8.2), 154 mM NaCl, 1% Triton X-100, 12 mM Na-deoxycholic acid, 1 mM naphtylphosphate, 10 mM EDTA, 0.2% NaN_3, 5 µg/ml leupeptin, 1 mM NaF, 2.5 mM β-mercaptoethanol, 0.1% polyvinylpyrrolidon K30 (PVP) and 5% PVPP. eIF-4A was immunoprecipitated using 10 µl eIF-4A antibody and 10 µl Pansorbin cells (Calbiochem) as described by Harlow and Lane (32). The precipitate was dissolved either by boiling in SDS–PAGE loading buffer for SDS–PAGE gel electrophoresis or in 100 mM triethylamine (pH 11.2) for loading at the basic side of an IEF tube gel.

Phosphoamino acid determination

eIF-4A proteins, immunoprecipitated from pollen germinated in medium containing $^{32}P_i$, were separated by SDS–PAGE and blotted onto PVDF membrane (Bio-Rad). After autoradiography, the radioactively labelled eIF-4A was excised and hydrolysed in 6 N HCl for 2 h at 115°C. After addition of 5 nmol phosphorylated serine, tyrosine and threonine the sample was vacuum dried. The pellet, containing ~200 d.p.m., was dissolved in 2 µl of running buffer (pH 1.9) and separated by two-dimensional thin layer electrophoresis on cellulose plates (Merck) according to Boyle *et al.* (33). The first dimension was run for 25 min at 1.3 kV using a buffer of pH 1.9 [formic acid (88%):glacial acetic acid:H_2O = 25:78:897]; the second dimension was run for 16 min at 1.3 kV using a buffer of pH 3.5 (glacial acetic acid:pyridine:H_2O =50:5:945). After electrophoresis, the plates were stained with 0.2% ninhydrin in acetone and autoradiographed.

In vivo expression of eIF-4A8

The *NeIF-4A8* genomic clone (in pBluescript SK) (accession number X79005), containing the 2137 bp upstream region responsible for the pollen specific expression (14), was used to construct *eIF-4A8* containing a his-tag (*eIF-4A8his*). A primer 5′-CGGGATCCTCA$(ATG)_6$GAGGAGATCAGCAACATTTGA-GGGG-3′ was used to introduce six histidines at the 3′-end of the *eIF-4A8* open reading frame by PCR, using a second internal primer at position 3310. The 3′-UTR of *eIF-4A8* was replaced by the *nos* 3′-terminator (34). The promoter activity of *eIF-4A8* is independent of its 3′-UTR (14). Leaves were transformed using vector pMON505 with the *Agrobacterium tumefaciens* strain LBA4404 as described by Draper *et al.* (35). The recombinant protein containing the his-tag (eIF-4A8his) was extracted from pollen in 1× binding buffer [5 mM imidazole, 500 mM NaCl and 20 mM Tris–HCl (pH 7.9)], 0.1% PVP, 5% PVPP and 0.1% Triton X-100, washed twice with 5% PVPP in the same buffer and purified with His-Tag binding resin in batch according to the pET System Manual (Novagen). The resin was washed twice with 1× binding buffer and twice with 32.5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl (pH 7.9). eIF-4A8his was eluted with 1 M imidazole, 500 mM NaCl and 20 mM Tris–HCl (pH 7.9) and precipitated with 5 vol acetone, with 15 µg rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Sigma) and 15 µg soybean trypsin inhibitor (Boehringer Mannheim) as carriers.

RESULTS

eIF-4A is phosphorylated in tobacco pollen

Since translational control is thought to be of prime importance during pollen tube germination (21,26), we were interested to investigate the phosphorylation status of eIF-4A during this developmental stage. Dry pollen was suspended in a synthetic medium allowing pollen tube germination in the presence of $^{32}P_i$. After various time points, eIF-4A was isolated by immuno-precipitation and visualized by SDS–PAGE and autoradiography (Fig. 1A). After 60 min, a signal could be observed at ~46 kDa, the size of eIF-4A. However, phosphorylation of eIF-4A may occur much earlier, considering the time delay caused by uptake, dilution by the internal phosphate pool and incorporation of $^{32}P_i$ in $AT^{32}P$ (or $GT^{32}P$). Therefore, it appears conceivable that phosphorylation of eIF-4A takes place soon after pollen hydration. Phosphorylation of eIF-4A in tobacco pollen confirms and extends results by previous workers, who also observed phosphorylation of plant eIF-4A (10,11). The immunoprecipitated phosphorylated eIF-4A was cut from a PVDF membrane and hydrolysed as described in Materials and Methods. Two-dimensional separation of the phosphoamino acids by TLC-electrophoresis showed that eIF-4A is phosphorylated primarily on a threonine residue or

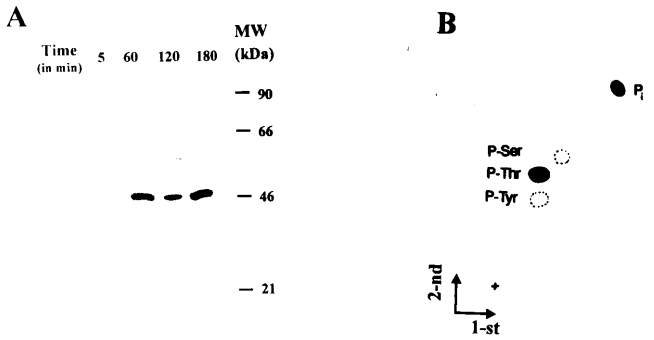

Figure 1. Analysis of eIF-4A phosphorylation. (**A**) Autoradiogram of immunoprecipitated eIF-4A which was separated by SDS–PAGE and subsequently blotted on a nitro-cellulose membrane. Pollen was incubated in germination medium containing 500 µCi $^{32}P_i$/ml for the times indicated before extracting protein. (**B**) After hydrolysis of immunoprecipitated eIF-4A, the phosphorylated amino acids were separated by two-dimensional TLC-electrophoresis.

residues (Fig. 1B). Longer exposure showed a weak signal at the position of phosphoserine as well (result not shown).

Pollen contain at least two phosphorylated isoforms

Pollen of tobacco contain at least three different eIF-4A mRNA transcripts, one of which is pollen specific (14). To identify which of the multiple isoforms is phosphorylated, eIF-4A was immunoprecipitated from pollen germinated in a medium containing $^{32}P_i$. After two-dimensional gel electrophoresis and western blotting, the phosphorylated isoforms were visualized by autoradiography (Fig. 2). Six different isoforms could be separated in extracts from germinated pollen (see also Fig. 3). The different isoforms fall into three groups: isoforms 1 and 2 have the lowest mobility, isoform 3, 4 and 5 have an intermediate MW and isoform 6 has the lowest MW. Comparison of the western blot with the corresponding autoradiogram shows that two of the isoforms, namely isoforms 1 and 3, have incorporated $^{32}P_i$ and thus represent phosphorylated forms of eIF-4A. These isoforms have the lowest pI, as could be expected. Since tobacco contains multiple transcripts of eIF-4A, it is not possible to conclude that the proteins with the same size (i.e. isoforms 1 and 2 or isoforms 3 and 4) arise by phosphorylation of the same protein.

eIF-4A is phosphorylated during pollen germination

We wanted to establish if the phosphorylation status of eIF-4A changes during pollen germination. The only way to label maturing pollen *in vivo* is to feed whole plants with radioactive phosphate. This is a rather cumbersome method and moreover, because internal phosphate pools may change during germination, changes in signal intensity may not reflect changes in phosphorylation status. By comparing western blots from germinating pollen with the corresponding autoradiograms, it is possible to identify the phosphorylated isoforms of eIF-4A. In Figure 2A, spots 1 and 3 represented phosphorylated eIF-4A (see above). Now it became possible to assess the changes in phosphorylation of eIF-4A by two-dimensional gel electrophoresis/western analysis with total protein extracts (Fig. 3). In dry mature pollen, only a small amount (~1–3% of total eIF-4A) of the phosphorylated eIF-4A isoform 1 was present, whereas isoform 3 was entirely absent. After 2.5 h of germination, there are approximately equal amounts of

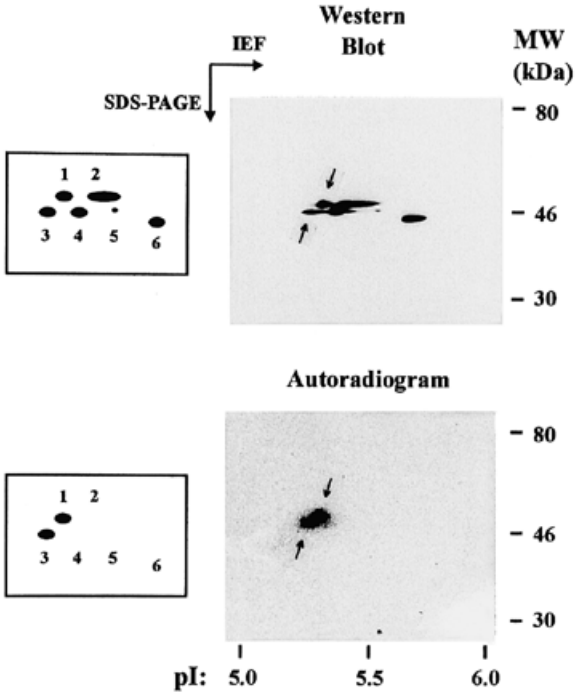

Figure 2. Determination of radioactively labelled eIF-4A isoforms. Protein was extracted from pollen which was germinated in medium containing 500 µCi $^{32}P_i$/ml. eIF-4A was isolated by immunoprecipitation, separated by two-dimensional electrophoresis/western analysis and visualized using immunodetection (**top**). The western blot was used for autoradiography to detect the radiolabeled isoforms (**bottom**). Schematic diagrams are shown on the left. The individual isoforms are numbered 1–6. The arrows indicate the radiolabeled isoforms.

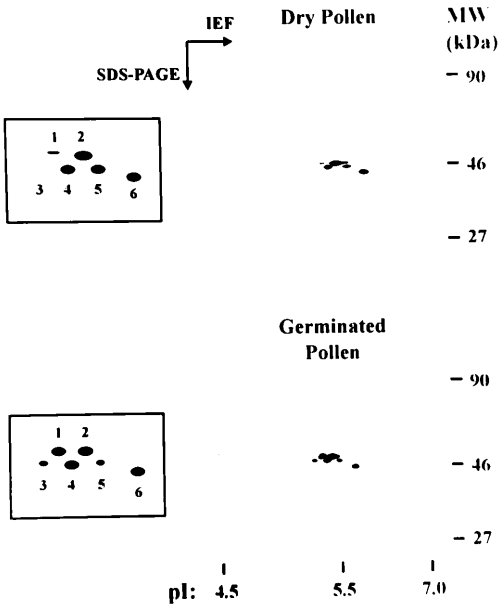

Figure 3. Two-dimensional gel electrophoresis/western analysis of eIF-4A from dry and germinated pollen. Samples (10 µg) of total protein were separated by two-dimensional gel electrophoresis, blotted onto nitro-cellulose and eIF-4A isoforms were visualized by immunodetection. The individual isoforms are numbered 1–6 as in Figure 2.

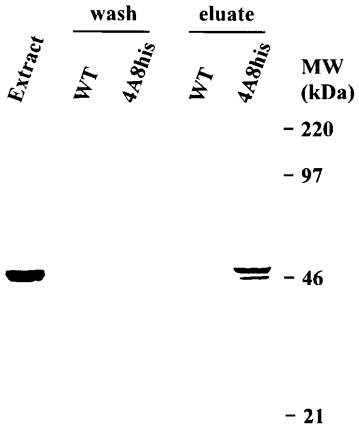

Figure 4. eIF-4A8his expression in transgenic plants. Total protein extracts (2.5 mg) of wild-type and transgenic pollen were incubated with his-tag binding resin, washed with a buffer containing 32.5 mM imidazole, 500 mM NaCl, 20 mM Tris–HCl (pH 7.9), and histidine-tagged proteins were eluted with the same buffer containing 1 M imidazole. From the total extract 1/250 part was loaded (wild-type and transgenic pollen extracts show exactly the same eIF-4A pattern), from the wash and the eluate 1/10th was loaded on an SDS–PAGE gel. eIF-4A was visualized on a western blot by immunodetection.

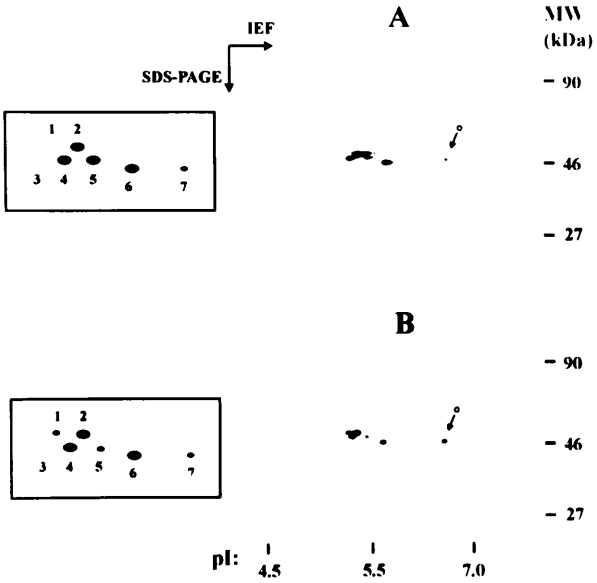

Figure 5. Identification of eIF-4A8 in wild-type dry pollen. Two-dimensional gel electrophoresis/western analysis of 10 µg total protein from (**A**) eIF-4A8his transgenic pollen, (**B**) 10 µg total protein from wild-type pollen mixed with isolated eIF-4A8his (1/10th of the eluate). The arrows with the open circle point toward eIF-4A8his. This isoform is indicated as number 7 in the diagram.

phosphorylated isoform 1 and non-phosphorylated isoform 2. The amount of phosphorylated isoform 3 was found to be 25–35% compared to isoform 4. We conclude that in mature pollen hardly any eIF-4A is phosphorylated and that during pollen tube growth ~15–20% of total eIF-4A becomes phosphorylated.

Transgenic plants overexpressing eIF-4A8 containing a histidine-tag

We showed that pollen contain multiple isoforms of eIF-4A, two of which are phosphorylated. However, the complexity of the eIF-4A gene family makes it hard to ascertain which gene product becomes phosphorylated. In particular, we would like to know whether the pollen-specific eIF-4A8 is phosphorylated and whether its phosphorylation status changes during pollen tube germination. To be able to study this specific isoform *in vivo* we generated transgenic plants expressing eIF-4A8 containing a histidine-tag at the C-terminus (eIF-4A8his). Total protein from pollen from wild-type and transgenic plants were extracted and histidine-tagged proteins were isolated with His-Tag binding resin. After separation on a 10% SDS–PAGE gel and western blotting, a signal was obtained from transgenic pollen, but not from wild-type pollen (Fig. 4). Thus, we were able to isolate eIF-4A8his free from other eIF-4A isoforms. To study eIF-4A8his expression in transgenic pollen, total protein extracts from eIF-4A8his overexpressing pollen were separated by two-dimensional gel electrophoresis and western blotted. One additional isoform appeared compared to wild-type pollen extracts (Fig. 5A, the additional isoform is indicated by an arrow with an open circle). The additional isoform has a pI near the predicted pI for eIF-4A8his (pI = 6.45). In the diagram, the additional isoform is indicated as isoform 7. To confirm that this isoform is eIF-4A8his, we mixed wild-type protein extracts with isolated eIF-4A8his. After two-dimensional gel electrophoresis and western blotting, the same pattern was obtained (Fig. 5B) as in transgenic pollen (Fig. 5A). From this we conclude that isoform 7 represents eIF-4A8his.

The transgenic plant approach enabled us to specifically study eIF-4A8 and determine whether the phosphorylation status of this isoform changes upon pollen tube germination. From both dry and germinated pollen, eIF-4A8his was isolated by affinity chromatography and separated by two-dimensional gel electrophoresis/western analysis (Fig. 6A and B). The results show that in dry pollen isoform 7 is the major eIF-4A8his. During pollen tube germination, a second eIF-4A8his isoform, isoform 8, becomes more apparent. Upon germination, the ratio of isoform 7 to isoform 8, both being eIF-4A8his isoforms, increased to near 50%. To identify phosphorylated isoforms, we isolated eIF-4A8his from pollen germinated in a medium containing $^{32}P_i$. After two-dimensional gel electrophoresis and western blotting, the phosphorylated isoforms were visualized by autoradiography (Fig. 6C). This clearly shows that isoform 8 is phosphorylated. Due to the fact that both isoforms arise from one gene, we conclude that isoform 8 is the phosphorylated form of isoform 7. The phosphorylated isoform becomes more prevalent upon pollen tube germination.

DISCUSSION

In this report we have shown that at least two isoforms of eIF-4A are phosphorylated in tobacco pollen, and one of them is the pollen-specific eIF-4A8. An increase in the phosphorylation status of eIF-4A was observed during pollen tube germination, a developmental stage characterized by extraordinarily high growth rates and hence high translational activity (21,27). So far, eIF-4A phosphorylation has only been documented for plants (10,11), and thus a function in a plant-specific process seems plausible. We suggest three possible functions for eIF-4A phosphorylation in pollen. First, eIF-4A phosphorylation might activate protein synthesis during pollen tube germination, i.e. when the demand for new proteins is very high. In this case, phosphorylation

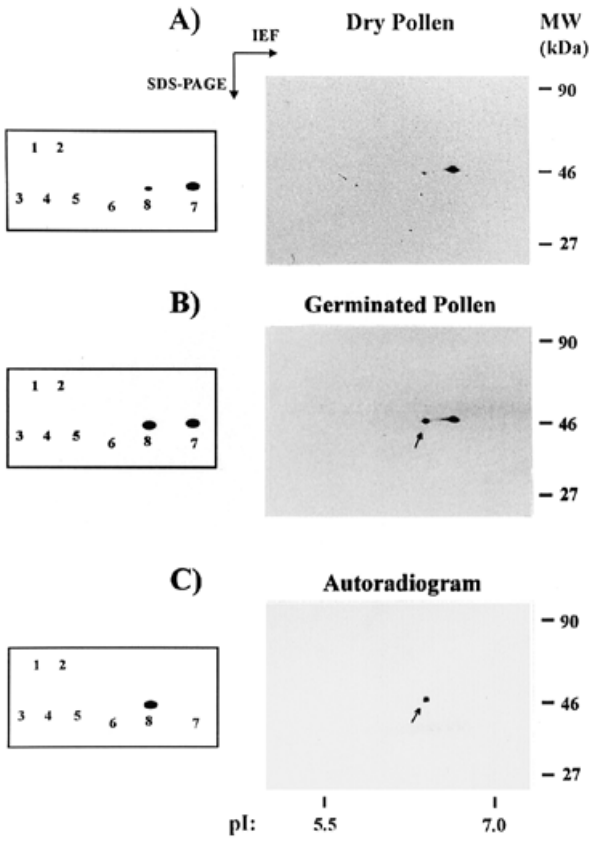

Figure 6. Determination of eIF-4A8his in dry and germinated pollen. eIF-4A8his was isolated with His-Tag resin from (**A**) dry and (**B**) germinated pollen. The protein was analysed by two-dimensional electrophoresis and western blotting. (**C**) Protein was extracted from pollen which were germinated in medium containing 500 µCi $^{32}P_i$/ml. eIF-4A8his was isolated with His-Tag resin and separated by two-dimensional electrophoresis/western analysis. The western blot was used for autoradiography to detect the radiolabeled isoforms. The arrow indicates the radiolabeled isoform, indicated as number 8 in the diagram.

would be expected to activate protein synthesis. Second, it could enable the translation of mRNAs during dehydration, when a concentration of KCl, estimated to be ~300 mM, would preclude normal protein synthesis. In particular, the stabilization of secondary RNA structures due to these high salt concentrations might require additional unwinding activity (26,30). This activity could be provided through regulation of eIF-4A activity. After pollen hydration, conditions return to more physiological levels and a fraction of eIF-4A would not be required. The excess of eIF-4A could be inactivated by phosphorylation. In this case, the non-phosphorylated form would be the active form, similar as proposed for eIF-4A during protein synthesis in hypoxic maize roots (10) and in heat shock treated wheat seedling leaves (11). Third, eIF-4A8 might play a role in the translation of pollen specific mRNAs such as the *lat52* mRNA. eIF-4A8 could be recruited into *lat52* specific initiation complexes. The 5′-UTR of *lat52* increases translation efficiency during pollen maturation when eIF-4A8 is poorly phosphorylated. Inactivation of eIF-4A8 by phosphorylation could reduce the synthesis of *Lat52* after a burst of synthesis during pollen tube germination. For *Xenopus* embryos, it was also suggested that a specific eIF-4A, eIF-4AIII, can regulate changes in cell fate through selective mRNA translation (36). In order to test these hypotheses it will be necessary to develop pollen-based *in vitro* and *in vivo* systems in which the function of (non-)phosphorylated eIF-4A can be studied.

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